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DETERMINATION OF ETHANOL PARTITION COEFFICIENTS TO THE INTERIOR AND THE SURFACE OF DIPALMITYL-PHOSPHATIDYLCHOLINE LIPOSOMES USING DEUTERIUM NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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The binding of ethanol-d_6 to dipalmityl-phosphatidylcholine liposomes (DPPC) can be separated into two processes, namely, ethanol in the bilayer and on the surface of the bilayer. For the deuterons of the methylene group, the T_2 of both bound states is shorter than the respective preexchange lifetime (τ_B) and therefore the amount of ethanol bound to both sites can be determined from the decrease in the methylene intensity resonance in the presence of DPPC. For the methyl resonance, however, only the T_2 of deuterons on ethanol bound to the surface is less than its τ_B and the amount of surface bound ethanol-d_6 can be determined. Subtraction yields the amount of ethanol bound within the bilayer. The partion coefficient for internally bound ethanol remains constant from 0 to 3.5 m ethanol. Surface binding is, however, highly cooperative.

There is now a wide variety of biophysical evidence supporting the lipid perturbation hypothesis of ethanol action (1). To understand the precise mechanisms and sites of the perturbation, it is necessary to investigate the membrane distribution of ethanol. In this regard, there have been numerous studies (2-4) in which the membrane partition coefficient of ethanol has been determined. These studies suggest that at low ethanol concentrations (~.4 m) ethanol partitions to the hydrophobic membrane core and the partition coefficient is, as expected, concentration independent. At higher concentrations, there is significant binding to the membrane surface which appears co-operative. Unfortunately, due to the nature of the methods by which K has been determined, the accuracy of these determinations must be questioned because of the numerous assumptions (4) and experimental difficulties inherent in the techniques used (5).

The partition coefficients of ethanol-d_6 in the presence of dipalmityl-phosphatidylcholine (DPPC) liposomes have been determined utilizing ^2H NMR. The utility of NMR in measuring the degree of binding of small molecules to macromolecules has been clearly

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demonstrated (6). If the transverse relaxation time in the bound state (T_{2B}) of the small molecule is short compared to the preexchange lifetime (τ_B), then the process is slow on the NMR time scale and the bound state is usually not detected due to line broadening. The determination of the observed spectral intensity then yields the partition coefficient directly.

MATERIALS AND METHODS

Materials: The DPPC was obtained from Sigma Chemical Co., St. Louis, MO. Gas chromatographic analysis (7) revealed the DPPC to be more than 99.5% pure. Ethanol-d_6 (density = 0.918/ml) was obtained from Cambridge Isotope Laboratories, Woburn, MA. Liposomes were prepared from the DPPC as described elsewhere (8) in phosphate buffered saline, pH = 7.6 at 22°C. Spectra were obtained at 46°C and thus the lipid was in the liquid-crystalline state.

Methods: The ^2H NMR spectra were obtained on a Nicolet NMC 300 FT-NMR spectrometer. The lock coil of 5 mm ^1H proton probe was used as the observe coil. The magnetic field strength was 7.05 Tesla with a frequency of 46.066 MHz for ^2H. The temperature was maintained at 46°C using a NTC temperature control unit. The spectrometer was run in the unlocked mode. The spectra were time averaged for 128 to 512 scans depending on the ethanol-d_6 concentration. Peak intensities were obtained using minimum Chi square residual fit of each resonance to a Lorentzian line, and peak area was taken as height times linewidth.

RESULTS AND DISCUSSION

The partition coefficient of ethanol-d_6 to the DPPC liposomes can be determined by ^2H NMR spectroscopy in the following manner. The addition of ethanol-d_6 to H_2O will result in the exchange of the hydroxyl deuteron with a proton to yield stoichiometric amounts of HOD and CD_3CD_2OH molecules. The exchanging ethanol species between the free and bound states will, therefore, be CD_3CD_2OH. The observed ^2H spectrum will consist of three resonances, namely, the HOD, CD_2 and CD_3 resonances. The intensity of the HOD will be the sum of the natural deuterium concentration (i.e. $[HOD]_{nat} = 0.01776$ m) and the concentration of added ethanol $[EtOH]_T$. Because exchange between the free and the bound states is slow on the NMR time scale and the bound state is broadened beyond detection, the intensity of the methylene group of the ethanol would be twice the added ethanol concentration minus twice the bound concentration $[EtOH]_B$. The ratio of the methylene to HOD integrated intensities is given by

$$R = \frac{2([EtOH]_T - [EtOH]_B)}{[HOD]_{nat} + [EtOH]_T} \quad (1)$$

which can be rearranged to

$$[EtOH]_B = [EtOH]_T - \frac{[HOD]_{nat} + [EtOH]_T}{2} \quad (R) \qquad (2)$$

Using the partition constant

$$K = \frac{[EtOH]_B}{[EtOH]_F} \qquad (3)$$

where $[EtOH]_F$ is the free ethanol concentration and is given by

$$[EtOH]_F = [EtOH]_T - [EtOH]_B = \frac{[HOD]_{nat} + [EtOH]_T}{2} \quad (R) \qquad (4)$$

Substitution of 2 & 4 into 3 and rearrangement gives,

$$K = \frac{2[EtOH]_T}{[HOD]_{nat} + [EtOH]_T} \left(\frac{1}{R}\right) \quad -1$$

Therefore, the K can easily be calculated from the ratio of the integrated intensities of CD_2 and HOD resonances. A similar expression for K derived from the intensities CD_3 and HOD can also be derived with the 2 replaced by a 3.

The concentration dependence of K as obtained from the intensities of the CD_2 and CD_3 resonances is shown in Figure 1. Both values show a concentration dependence and the K derived from the CD_2 resonance being greater than that from the CD_3 resonance by a constant amount.

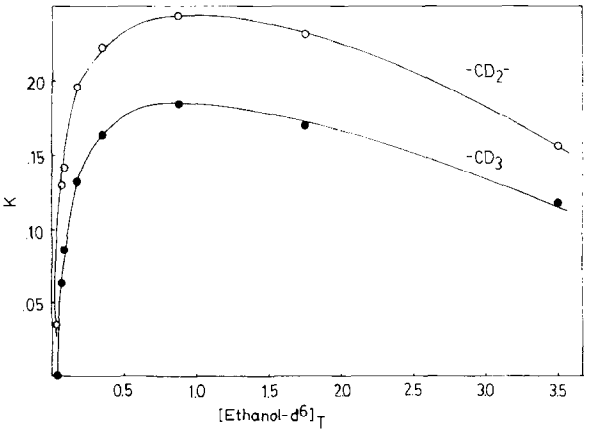

Figure 1 - The molar concentration dependence of the partition coefficient of ethanol-d_6 with DPPC liposomes at 46°C as determined from the decrease in intensity of the CD_2 and CD_3 resonances. The DPPC concentration is 0.4% by weight in H_2O-PBS buffer.

It must be remembered that for the exchange of a small molecule to the membrane to be slow on the NMR time scale, the $1/T_2$ of the bound state must be greater the inverse of the pre-exchange lifetime τ_B (6). Because of greater freedom of rotation of the methyl group, the T_2 of CD_3 is usually longer than for the CD_2 group (9). Therefore, because of this difference in T_2's, for the same exchange process, the NMR time scale for the CD_3 resonance can be fast but slow for the CD_2 resonance.

The difference in binding constants can be interpreted as follows. There are two distinct binding sites for the ethanol on the membrane each with its unique relaxation time (T_{2B_1} and T_{2B_2}), unique preexchange lifetime (τ_{B_1} and τ_{B_2}) and unique binding constant (K_1 and K_2). Because of its shorter T_2, the NMR time scale for the CD_2 resonance is slow for exchange between the bound state and the free. The K determined by this resonance is the overall binding constant of ethanol to membrane as observed by this method and is the sum of K_1 and K_2 for each site. For the CD_3 resonance, however, its T_{2B_1} in one bound state is fortuitously longer than τ_{B1} and fast exchange is observed. The T_{2B_2} of the other bound state is shorter than τ_{B2} and slow exchange is observed. The reduction in intensity of the CD_3 resonance can, then be used to obtain K_2. Substraction of K_2 from K, yields K_1. These results are summarized in Table I.

Tentatively, we assign K_1 as the partition coefficient of ethanol to the membrane interior. It is constant over a wide concentration range which is consistent with

TABLE I

PARTITION COEFFICIENTS FOR ETHANOL -d_6 WITH DPPC LIPOSOMES AT 46°C

[Ethanol -d_6]	K_1	K_2
0.035 m	.0661	-.0312
0.070 m	.677	.0630
0.105 m	.0553	.0867
0.175 m	.0657	.1307
0.350 m	.0590	.1630
0.875 m	.0599	.1841
1.75 m	.0612	.1698
3.5 m	.0397	.1168

K's are expressed in terms of moles bound/moles free. Conversion to K's in terms of molality bound/molality free can be obtained by multiplying these values by 250.

partitioning to hydrophobic site(s). Furthermore, the values obtained are similar to those found with other methods which are thought to reflect partitioning to the hydrophobic membrane interior (4). The cooperative nature of the binding to site-2 (as reflected by the increasing value of K_2) may represent surface binding of the type described by Rowe (4). K_2 may be cooperative in nature because of a gradual melt of the "ice" like water on the bilayer surface with the presence of increasing ethanol.

The utility of ^2H NMR to ascertain the existence of different kinds of binding sites of ethanol on DPPC bilayers is clearly demonstrated. Further work is currently progressing to determine the exact nature of the binding sites and if similar sites exist on natural membranes.

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